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# ENHANCED PHAGOCYTOSIS OF **SALMONELLA ENTERITIDIS** WITH HYPOAGGLUTINATING MOUSE ANTISERA

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It has been well documented that in humans a typhoid infection due to **Salmonella typhosa** is accompanied by the formation of serum antibodies which will agglutinate the bacteria **in vitro.** Furthermore the presence of the agglutinins has been correlated with resistance to the disease. Animal (e.g. rabbit) inoculation with the organism **S. typhosa** also results in the production of serum agglutinins. It was with some surprise, then, that Paulissen and Shechmeister (9) discovered that mouse inoculation with **Salmonella enteritidis** was not followed by production of agglutinins in amounts usually considered significant. The surprise was even greater when Hatch (4) showed that agglutinins were produced in mice against another Gram-negative organism **Proteus morganii** and that these agglutinins were protective. Gorer and Schůtze (3) while able to demonstrate agglutinins against **S. enteritidis** in mice were unable to correlate resistance of the mice with them. The apparent differences in all these responses could be due to differences among the species and, regarding mice in particular, differences among the strains.

With respect to the particular strain of mice (NAMRU) and strain of **S. enteritidis** (1891) that Paulissen and Shechmeister worked with (9) it was speculated that either the standard agglutination test was inadequate or sensitive enough, or, that perhaps another kind of immune mechanism may be operating. The work presented here shows that protective factors are present in sera of mice and that they, or others, enhance phagocytosis of the organism even though no "significant" agglutinin titer could be demonstrated.

## MATERIALS AND METHODS

**Experimental animals:** — Ten-to-sixteen week old male and female NAMRU (2) strain mice were used in this study. The mice, caged in glass jars, were fed Ralston-Purina Laboratory Chow exclusively and provided water, both **ad libitum.**

**Test organism and preparation of vaccine:** — Growing the organism for challenge and the preparation of the vaccine was done in essentially the same manner as previously described (9). The organism was **Salmonella enteritidis** 1891 (IX, XI . . . g,m) and originally came as culture 64 of the University of Kentucky. It was stored in ampoules as noted in (9).

**Antisera:** — Using two groups of mice, two separate pools of antisera were prepared at different times. No difference of reactability was detected between the separate pools of sera. Each mouse received

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three injections on alternate days during a six-day period. Each injection consisted of 0.2 ml of vaccine containing approximately $4.4 \times 10^8$ organisms, making a total of 0.6 ml of vaccine per mouse. Fifteen to sixteen days elapsed between the last immunizing dose and the collection of the antisera.

Collecting the blood was done by lightly anaesthetizing the mice with ether, then, severing the jugular vein with dressing scissors, allowing the blood to drop into a small test tube. Between 1.0 and 1.2 ml of blood per mouse were obtained in this manner. Pooled blood was held at room temperature for two hours after which time it was centrifuged and the sera drawn off. The immune sera were stored in sterile screw-capped test tubes at 4-6°C.

## PROCEDURES AND RESULTS

Before attempting to determine the presence of antibodies in mouse sera it was decided first to see if such sera conferred protection. A group of six mice was passively immunized with "immune" mouse sera, 0.2 ml intraperitoneally per mouse, and another group of six was given sera from non-immunized mice. Each group was challenged intraperitoneally with approximately $4.4 \times 10^8$ organisms contained in 0.2 ml saline ($LD_{50}$ previously determined to $4.4 \times 10^{6.3}$) 30 minutes after

Table I.

Results of Passive Immunization of Mice Against
**Salmonella entertidis**

| Group No. | Treatment (0.2 ml,i,p) | Day of Death After Challenge* | Dead / Total | Mean Survival Time |
|---|---|---|---|---|
| I | Undiluted Mouse Antisera | 1,1,4,20,28,28 | 6/6 | 13.6 |
| II | Normal Mouse Sera | 1,1,1,1,2,2 | 6/6 | 1.6 |

*Challenged with 0.2 ml of approximately $4.4 \times 10^8$ organisms i.p., 30 minutes after treatment.

treatment. Results are shown in Table I. It can be seen that in terms of survival time a mean of 13.6 days for those receiving immune sera was obtained compared to a survival time mean of 1.6 days for those mice receiving nonimmune sera. Thus the sera from immunized mice conferred protection.

Separate standard agglutination tests were then performed on sera from ten different immunized mice. No agglutinins were observed in any of them at a titer of 1:20 or more. It was concluded that routine procedures were inadequate for demonstrating agglutinating antibodies, if present.

A series of agglutination tests was then performed to test specifically for "H" and "O" agglutinins. Antigens were prepared as follows according to procedures described by Kolmer et al (7). The "O" antigen was prepared by adding 2-3 ml of 95% alcohol to 50 ml of a 24-hour tryptose broth culture of **S. enteritidis** and subsequently incubated at 37°C for 12 hours. The organisms were washed twice in normal saline and resuspended in fresh saline to a turbidity equal to the No. 2 tube of the McFarland nephelometer. The "H" antigen was prepared by adding 1 ml of 0.5% formalin to 50 ml of a 24-hour tryptose broth culture of the organism which was washed in normal saline twice and resuspended in fresh saline in amount to produce turbidity equal to the No. 2 tube of the McFarland nephelometer. The tube agglutination tests were conducted on pooled mouse antisera using routine procedures as above. The "O" antigen series were incubated in a water bath at 50°C overnight before reading. The "H" antigen series were incubated in a 50°C water bath for 2 hours after which time they were placed in the refrigerator overnight. An antiserum from a rabbit was also tested for comparison. The results of the tests (Table II) revealed that the "H" antigen produced a titer of 1:160, whereas the "O" antigen gave a titer of only 1:40 in the mice.

Table II.

Agglutination Tests on Mouse and Rabbit Antisera
Against "O" and "H" Antigens of
**Salmonella enteritidis**

| Anti-sera | Anti-gen | Reciprocal of Titer 20 | 40 | 80 | 160 | 320 | 640 | 1280 | Con-trol |
|---|---|---|---|---|---|---|---|---|---|
| Mouse | O | +++ | ++ | — | — | — | — | — | — |
| | H | ++++ | +++ | +++ | ++ | — | — | — | — |
| Rabbit | O | ++++ | ++++ | ++++ | ++++ | ++ | — | — | — |
| | H | ++++ | ++++ | ++++ | ++++ | ++++ | +++ | ++ | — |

— = No reaction
\+ = Degree of agglutination

**"H" Antigen Agglutination Test Using Special Conditions:** — Further agglutination tests under various conditions were conducted using the "H" antigen: it did produce the more sensitive agglutinating antibody reaction above. The conditions consisted of three different temperatures,

pH ranges, and levels of salt concentration. The temperatures were 22°C, 37°C, and 50°C. The pH ranges were 4.8, 6.8, and 7.8. The salt concentrations were 0.70%, 0.85%, and 1.0%. The "H" antigen was prepared from 50 ml of a 24-hour tryptose broth culture of the organism to which 1 ml of 0.5% formalin was added and incubated at 37°C for 12 hours. The tests were incubated for 2 hours at the temperatures indicated, followed by standing in the refrigerator overnight. Again, rabbit antiserum was also tested. The results are presented in Table III. It can be seen from the Table that the best agglutination in mouse antisera was obtained at an optimal condition of a temperature of 50°C, a pH of 6.8, and a salt concentration of 0.85%, i.e., the standard conditions routinely used in such tests.

Table III.

"H" Agglutination Tests Against Mouse Antisera Under Varying Conditions

| Test Sera | Condition variable | Conditions | | | Titer of Sera[1] |
|---|---|---|---|---|---|
| | | pH | %NaCl | Temp. | |
| Mouse Antisera | pH | 4.8 | 0.85 | 50°C | Neg. |
| | | 6.8 | 0.85 | 50°C | 1:160 |
| | | 7.8 | 0.85 | 50°C | 1:80 |
| | %NaCl | 6.8 | 0.7 | 50°C | Neg. |
| | | 6.8 | 0.85 | 50°C | 1:160 |
| | | 6.8 | 1.0 | 50°C | 1:80 |
| | Temp. | 6.8 | 0.85 | 22°C | Neg. |
| | | 6.8 | 0.85 | 37°C | 1:20 |
| | | 6.8 | 0.85 | 50°C | 1:320 |
| Normal Mouse Sera | | 6.8 | 0.85 | 50°C | Neg. |
| Normal Rabbit Serum | | 6.8 | 0.85 | 50°C | Neg. |
| Rabbit Antiserum | | 6.8 | 0.85 | 50°C | 1:1280 |

Readings were made after two hour incubation at the respective conditions and left to set at room temperature for 15 minutes.

[1]Reading of ++ or more.

**Blocking Test, "Incomplete Antibodies":** — So-called incomplete antibodies fail to agglutinate saline suspensions of homologous cells, but their presence can be detected by one or more methods. One kind of incomplete antibody can be demonstrated by the following type of "blocking test". **S. enteritidis** in saline suspension when mixed with the mouse antisera should fail to be agglutinated when subsequently exposed to homologous rabbit antisera. If blocking antibodies were present in the mouse antisera they would expectedly combine with the corresponding agglutinogen(s) of the bacteria and specifically prevent later reaction with the rabbit agglutinin.

The procedure for the "Blocking Test" performed is as follows. Beginning with a 1:10 dilution, serial dilutions of the mouse antisera were made and a routine agglutination test was conducted. The tubes were incubated for 12 hours in a 50° water bath; following the incubation period a reading was made to determine the amount of agglutination. The readings were recorded (Table IV) and 0.5 ml of a 1:160 dilution of rabbit antiserum added to each tube. The tubes were again incubated overnight in a 50°C water bath. The agglutination results are given in Table IV. The 4-plus reading in each tube indicates that incomplete "blocking" antibodies were lacking in the mouse sera.

Another method used to detect incomplete antibodies was the conglutination test. The procedure consists of a routine agglutination test except that Specific Bovine Albumin (Dade) was used in preparing the serial dilution instead of the usual normal saline. The negative readings (Table IV) indicate that no incomplete antibodies were present in the immune sera of the mice.

**Phagocytosis:** — It has been shown that unless certain types of Gram-negative bacteria are mixed with immune sera they are not readily phagocytosed by mouse macrophages (11). With this in mind it was decided to determine whether mouse antisera would affect the phagocytosis of **S. enteritidis** by mouse polymorpho nuclear leukocytes.

The production and collection of the phagocytes from the mouse peritoneal cavity was patterned after the method described by Fishman and Shechmeister (1). Mice were injected intraperitoneally with 1 ml of an Aleuronat suspension and sacrificed by severing the cervical vertebrae. The mice were dipped in 1:500 Roccal solution then the skin was reflected over the abdomen. Five-tenths ml of sterile 0.85% saline was introduced into the peritoneal cavity, mixed, and as much of the fluid removed as possible. The fluid containing the cells was immediately placed in heparinized capillary tubes and incubated at 37°C until used. Counts on the cell population showed from $3.5 \times 10^5$ to $4 \times 10^5$ cells per ml.

Using a modification of the technique described by Huddleson et al (5), 0.2 ml of bacteria was added to 0.2 ml of the test sera in serological tubes and placed in a 37°C incubator for 45 minutes. To this suspension 0.2 ml of the heparinized phagocytes was added and the mixture incubated at 37°C for one hour. With a Pasteur pipet a portion of the

Table IV.

Tests For Incomplete Antibodies In Mouse Antisera Against **Salmonella enteritidis**

| Anti-sera | Titer of Sera[1] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 20 | 40 | 80 | 160 | 320 | 640 | 1280 | 2560 | Con-trol |
| **BLOCKING TEST** | | | | | | | | | |
| Mouse | ++ | + | − | − | − | − | − | − | − |
| 50°C incubation overnight | | | | | | | | | |
| Rabbit | +++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | +++ | − |
| **CONGLUTINATION TEST** | | | | | | | | | |
| Mouse | − | − | − | − | − | − | − | − | − |
| Rabbit | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | − |

[1]Reciprocal of the dilution

− = No reaction

+ = Degree of agglutination

sediment was drawn off and smears were made. The smears were passed through an open flame 3 times to promote quick drying which minimizes shrinkage of the phagocytes.

The phagocytic index was calculated for the sera tested. For each test sera 25 polymorphonuclear leukocytes containing bacteria were counted on each of 4 different smears for a total of 100 cells. The index was obtained by dividing the total number of bacteria phagocytosed by the total number of phagocytes counted. The results are presented in Table V. Also the per cent of phagocytes containing bacteria was

Table V.

Phagocytic Indexes of Mouse and Rabbit Antisera Against **Salmonella enteritidis**

| Treatment of Bacteria with | Slide No. | Number of Phagocytes Counted | Number of Bacteria | Phagocytic Index |
|---|---|---|---|---|
| Mouse Antisera | 1 | 25 | 357 | 14.8 |
| | 2 | 25 | 393 | |
| | 3 | 25 | 345 | |
| | 4 | 25 | 386 | |
| Rabbit Antiserum | 1 | 25 | 314 | 12.3 |
| | 2 | 25 | 322 | |
| | 3 | 25 | 296 | |
| | 4 | 25 | 301 | |
| Normal Mouse Sera | 1 | 25 | 120 | 4.2 |
| | 2 | 25 | 102 | |
| | 3 | 25 | 101 | |
| | 4 | 25 | 96 | |

calculated (Table VI). From the above Tables it can readily be seen that the phagocytic index for both the mouse and the rabbit antisera is significantly higher than that of the normal mouse sera.

Table VI.

Number of Mouse Peritoneal Phagocytes Showing Phagocytosis

| Sera | Total Number of phagocytes counted | Number Showing Phagocytosis | Per Cent Showing Phagocytosis |
|---|---|---|---|
| Mouse antisera | 158 | 100 | 63.2 |
| Rabbit anti-serum | 172 | 100 | 58.2 |
| Normal Mouse Sera | 262 | 100 | 38.2 |

## DISCUSSION

The present studies have shown that there is something present in the antisera of immunized mice that can protect mice by prolonging the mean time to death from 1.6 days to 13.6 days (Table I). These results to some extent support Paulissen's hypothesis (9) that humoral elements play an important role in resistance of actively immunized, irradiated mice.

The agglutinating antibody is sometimes associated with resistance (10). This is especially true of the anti-"O" antibody (10). The present studies failed to show conclusively that an agglutinating antibody in the immune sera (Table II) is important. A titer of 1:40 was obtained against the "O" antigen and a titer of 1:160 was obtained against the "H" antigen. Ordinarily titers of this magnitude are not considered to be sufficient to produce protection. On the other hand, if one takes into consideration the size of the mouse as compared to the size of the rabbit or human, it seems feasible that these low titers may confer protection to the mouse. The studies on agglutination using various conditions failed to give an increase in titer over that of standard conditions.

The series of tests on the immune sera for incomplete antibodies failed to produce evidence of such an antibody (Table IV). Agglutination of the bacteria by the rabbit antiserum after no agglutination by the mouse antisera, indicates the organisms were not tied up by incomplete antibodies in the mouse antisera. The 3-plus reading in the first tube (Table IV) may be due to the slight agglutination (2-plus) by the mouse antisera. The absence of incomplete antibodies was also supported by the conglutination test performed (Table IV).

An **in vitro** experiment using mouse polymorphonuclear (PMN) leukocytes was conducted in this study. The results in Table V clearly show that the immune sera from both the mouse and the rabbit greatly enhance the phagocytosis of bacteria **in vitro** by the PMN leukocytes. Also it is shown (Table VI) that a greater number of PMN leukocytes phagocytose the bacteria in the presence of immune sera than normal sera. These findings are in agreement with others (6, 11) who have found that specific antibody enhances phagocytosis. Of some note is that the rabbit antiserum containing agglutinins to a titer of 1:1280 showed less ability to enhance phagocytosis by mouse leukocytes than the mouse antisera with virtually no agglutinin titer. This suggests either that rabbit agglutinating antibodies are considerably less effective in aiding mouse leukocytes than the meager mouse agglutinating antibodies, possibly resulting from complications due to species differences, or that agglutinating antibodies and those which enhance phagocytosis are of two different kinds in the mouse. It is tempting to consider the latter to be the more likely.

It is interesting to note in connection with the presence of anti-"H" antibodies (Table II) that Gorer and Schütze (3) found in a strain of mice resistant to **Salmonella typhimurium** that the specific antibody response to the "H" antigen was better than in a susceptible strain, but this relation did not hold for the "O" antigen. This appears strange since anti-"O" agglutinins are considered more important in resistance to Salmonellae than are anti-"H" agglutinins (10). The findings of Gorer and Schütze of a protective anti-"H" anti-body when considered together with results of Ralston and Paulissen (8) in which mouse antisera were found to inhibit motility of **S. enteritidis,** and the results herein showing enhancement of phagocytosis, suggest that inhibition of motility of **S. enteritidis** by mouse (anti-"H"?) antisera, that is, slowing the organisms down and/or immobilizing them, may well aid in their ingestion by the phagocytes and may be an important factor of immunity observed by Gorer and Schütze. This point was noted by William Champlin.*

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